Efficacy of Antifungal Agents in the Cornea

IV. Amphotericin B Methyl Ester

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Quantitative mycologic techniques were used to evaluate the efficacy of topical amphotericin B methyl ester in two models of yeast infection in rabbit eyes. Doses of 1%, 0.5%, and 0.15% were used in a model of superficial Candida albicans infection. The 1% dose of drug was highly efficacious, abolishing the disease after 2 days of treatment. With doses of 0.5% and 0.15%, decreasing efficacy was observed. Antifungal activity did not deteriorate when 1% prednisolone acetate was administered concomitantly with the 1% dose. In a model of deep stromal infection, the administration of topical 1% amphotericin B methyl ester was highly efficacious when the corneal epithelium was absent. Even in corneas with intact epithelium, a reduced though still significant effect was noted. Invest Ophthalmol Vis Sci 25:851–854, 1984

Ten years ago a group at Rutgers University, seeking to enhance amphotericin B, synthesized a methyl ester derivative.1 Although the new compound gave promise of improved efficacy by reason of its aqueous solubility and reduced toxicity, its potential as a topical agent in the eye has yet to be explored. In this paper, we describe our experimental studies with amphotericin B methyl ester in both superficial and deep infections of the cornea, including an evaluation of the influence of concomitantly administered corticosteroid on the efficacy of the drug and the barrier effect of the corneal epithelium.

Materials and Methods

Efficacy in a Model of Superficial Infection

A superficial infection with Candida albicans was established in the corneas of outbred pigmented rabbits, 1.5–3 kg in weight using a multiple trephination technique.2 Two-day-old cultures of C. albicans (strain LV) grown in trypticase soy agar with 5% sheep blood served as the inoculum. A suspension of 5 × 10⁹ colony forming units (CFU)/ml was prepared in normal saline and stored overnight prior to use. The LV strain has an MIC of 0.2 µg/ml and MFC of 0.78 µg/ml to amphotericin B methyl ester.2

Immediately after inoculation, the animals were allocated randomly into three groups—an untreated control group, a group treated with 1% amphotericin B methyl ester alone, and a third group treated with both 1% amphotericin B methyl ester and corticosteroid.

One hour after inoculation, treatment was begun. Amphotericin B methyl ester, prepared as a 1% solution in distilled water from the aspartate salt (E.R. Squibb & Sons; Princeton, NJ) was administered 10 times a day at hourly intervals. One percent prednisolone acetate (Pred Forte 1%, Allergan Pharmaceuticals; Irvine, CA) was given four times a day. The extent of the treatment period was either 1 day or 2 days. There were three animals in each group and both eyes of each animal received the same treatment.

Dose Response Studies

The same model of infection was used to study the efficacy of amphotericin B methyl ester administered in a concentration of 0.5% and 0.15%. Immediately after inoculation animals were separated randomly into three groups: untreated controls, a second group treated with 1% amphotericin B methyl ester alone, and a third group treated with both 1% amphotericin B methyl ester and corticosteroid.

One hour after inoculation, treatment was begun. Amphotericin B methyl ester, prepared as a 1% solution in distilled water from the aspartate salt (E.R. Squibb & Sons; Princeton, NJ) was administered 10 times a day at hourly intervals. One percent prednisolone acetate (Pred Forte 1%, Allergan Pharmaceuticals; Irvine, CA) was given four times a day. The extent of the treatment period was either 1 day or 2 days. There were three animals in each group and both eyes of each animal received the same treatment.

Influence of the Corneal Epithelium on Efficacy

For this experiment, an infection was established in the corneal stroma of outbred pigmented rabbits 1.5–
3 kg in weight using the LV strain of *C. albicans*. Rabbits were anesthetized with intramuscular ketamine hydrochloride and xylazine hydrochloride. A retrobulbar injection of 1% xylocaine was given. Topical anesthesia was achieved with Alcaine 0.5%. The eye was proposed gently and, using the operating microscope for visualization, a 30-gauge needle attached to a 250-μl Hamilton gas-tight syringe was introduced into the corneal stroma 2 mm from the limbus and advanced to the central cornea. Twenty-five microliters of the yeast suspension in a concentration of $5 \times 10^9$/ml were injected, and the needle was removed. If penetration of the anterior chamber occurred, the animal was removed from the study.

Following inoculation, 12 animals were allocated randomly to three equal groups. In the first two groups, the corneal epithelium was left intact; in the remaining animals, a 7-mm disc of central epithelium was marked with a disposable trephine and gently removed by scraping with a #15 Bard Parker blade (Bard Parker Co; Rutherford, NJ). The first group served as untreated controls. In the second and third groups, both eyes of each animal were treated with amphotericin B methyl ester 1% in distilled water. Beginning 1 hr after inoculation, the agent was administered hourly 10 times a day for 5 days. In the debrided epithelium group, the corneal epithelium was removed each day. In the intact epithelium group the rabbit corneas were inspected carefully for evidence of epithelial loss.

**Isolate Recovery**

In each experiment, 16 hr after the conclusion of treatment the animals were killed with commercially prepared euthanasia solution T-61 (Taylor Pharmacal; Decatur, IL). The whole corneas were removed by excision at the limbus and cut into small pieces. These were ground in a tissue grinder (Ultraturrax Model SDT; Tekmar Co.; Cincinnati, OH) for three 10-sec intervals in 3 ml of sterile phosphate buffered saline. One-, ten-, and one hundred-microliter aliquots of each corneal suspension were plated in triplicate on trypsinase soy agar with 5% sheep blood (BBL) and after 48 hr incubation at 25°C, the colony forming units (CFU) were counted, and the number of CFU per whole cornea was calculated based on a total volume of 3 ml.

**Statistical Analysis**

The measure of response to treatment was the logarithm to the base 2 of CFU. This transformation is required because the effect of treatment is proportional to pretreatment CFU. Since zero CFU counts were observed in some treatment groups, the actual response measure was $\log_2 (1 + \text{CFU})$. The rationale for the log transformation is discussed extensively by Snedecor and Cochran, and Steel and Torrie.

Three types of experimental designs were employed in this investigation. In the study of efficacy of the 1% solution in a model of superficial infection, there were three groups—control, drug alone, and drug with steroid—with three rabbits in each group. In the dose-response experiment, there were three groups—control, 0.15% drug, and 0.5% drug—with four rabbits in each group. Finally, in the study of the influence of the corneal epithelium, there were three groups—control, debrided epithelium, and intact epithelium—with four animals in each group. To achieve greater precision for one of the planned comparisons, the third experiment was replicated.

The data were analyzed using analysis of variance with subsampling. This technique recognizes the fact that the rabbit is the basic experimental unit; all conclusions are based upon the numbers of rabbits, not upon numbers of eyes. However, it utilizes the additional information from the two eye measurements to produce a more precise measurement of the effect of the treatment upon a single rabbit, as well as an estimate of the intrarabbit, intereye variability. This technique is described in detail in statistical texts.

Each hypothesis of interest was tested using a single degree of freedom contrast, which compared the salient experimental groups. For the replicated experiment, the contrast was constructed so that each experimental group was compared with the control from the same experiment. For example, intact epithelium group (EPION) versus control (CON) was tested with a contrast of the form:

$$ (\text{EPION}_1 - \text{CON}_1) + (\text{EPION}_2 - \text{CON}_2). $$

In this notation, the subscript “1” denotes the first performance of the experiment; “2” the second performance.

The alternative of simply pooling the data is unacceptable because the level of disease in the control group may be different for different replications of the same experiment.

For a single experiment, the estimated standard deviation of a contrast was derived from the between-rabbits mean square from the analysis of variance with subsampling. For the replicated experiment, it was derived from the between-rabbits (by replications) mean square. This accounted for the fact that rabbits were the basic experimental units, not eyes.

These investigations adhered to the ARVO Resolution on the use of animals in research.

**Results**

**Efficacy in the Model of Superficial Infection**

The 1% concentration of amphotericin B methyl ester was highly efficacious for superficial corneal infections at both 24 and 48 hr, abolishing the disease
at 48 hr (Fig. 1). The concomitant administration of 1% prednisolone acetate did not influence adversely the efficacy of the drug at either sampling point.

**Dose-Response Studies**

At lower doses of the drug (0.5% and 0.15%), a decreased response, as measured by numbers of organisms killed (relative to control) was seen (Fig. 2). For the 0.15% dose, an average of $2^{10}$ organisms were recovered from treated eyes, as compared with none for the 1.0% dose. (In both cases, approximately $2^{15}$ organisms were recovered from the untreated controls).

**Influence of the Corneal Epithelium on Efficacy**

In the model of deep stromal infection, the drug in the 1.0% dose was highly effective when the epithelium was debrided prior to treatment, virtually abolishing the disease (Fig. 3). However, even in corneas with intact epithelium, a significant therapeutic effect was noted. Relative to untreated controls, a nearly 13-fold reduction in number of organisms recovered occurred.

**Discussion**

There is good reason to consider amphotericin B methyl ester for use as a topical preparation in the eye. Its ready solubility in water, obviating the deoxycholate solubilizer, should make toxicity less of a problem. At the same time, the agent appears to have retained much of the broad spectrum antifungal activity of amphotericin B.3

Our dose response studies with amphotericin B methyl ester indicate that a concentration of 1% is required to eradicate completely the infection in 48 hr in this model. In previous experiments, a similar effect was noted with amphotericin B in the 0.075% and 0.15% concentrations.2 This reduction in potency is consistent with previous observations made with the systemic preparation by Gadebusch et al.6

The efficacy of most topical antifungal agents, with
the notable exception of amphotericin B, is affected adversely by concomitant steroid administration. Why amphotericin B should be exempt from this effect is not yet apparent, but strain susceptibility and a fungicidal rather than fungistatic mechanism of action may be important contributing factors. Our finding in the present study, that the efficacy of 1% amphotericin B methyl ester was likewise uninfluenced by concurrent steroid therapy, is further evidence that the agent has potent antifungal activity.

A major concern with the use of amphotericin B as a topical preparation has been the apparent lack of penetration into the cornea. We have shown recently that amphotericin B in concentrations of 0.15% and 0.075% is highly efficacious in experimental deep corneal infection provided the corneal epithelium is absent. However, even in the presence of this layer, a significant therapeutic effect was observed with the 0.15% preparation, though not with the more dilute concentration. Amphotericin B methyl ester behaves in a similar fashion. In the model of stromal infection, the 1% solution exhibits an efficacy roughly equivalent to that of 0.15% amphotericin B in corneas with intact and debrided epithelium. The hope that this soluble compound might demonstrate some improved ability to penetrate the epithelial layer was not realized in these studies. The epithelium also constitutes a partial barrier to the penetration of amphotericin B methyl ester.

These observations suggest that amphotericin B methyl ester has potential for topical use. They are of particular significance in view of the dearth of efficacious topical antifungal agents. However, an evaluation of toxicity, particularly systemic side-effects, and further experience with other strains are needed before a clearer view of the agent can emerge.

These data also provide additional evidence of the antifungal potency of the amphotericin B molecule. Our studies with both amphotericin B and its methyl ester derivative have been conducted with topical formulations empirically prepared from drug intended for systemic use. We believe that efforts should now be directed towards the development of improved formulations, perhaps by the use of such techniques as liposomal incorporation, that would be relatively nontoxic, while at the same time retaining a broad spectrum of antifungal activity.

Key words: Candida albicans, amphotericin B methyl ester, prednisolone acetate, corneal epithelium, efficacy

References